

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6: C12N 15/82, 15/53, A01N 63/00, A01H 5/00	A1	(11) International Publication Number: WO 95/14784 (43) International Publication Date: 1 June 1995 (01.06.95)
(21) International Application Number: PCT/US94/11837 (22) International Filing Date: 25 October 1994 (25.10.94) (30) Priority Data: 08/161,041 24 November 1993 (24.11.93) US (71) Applicant: MONSANTO COMPANY [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US). (72) Inventors: LAWRENCE, Ellen, Briakin; 219 North Mosley Road, Creve Coeur, MO 63141 (US). LEVINE, Elaine, Beatrice; 12392 Eastland Court, St. Louis, MO 63146 (US). SHAH, Dilipkumar, Maganlal; 14800 Lupine Court, Chesterfield, MO 63017 (US). (74) Agent: BOLDING, James, Clifton; Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GR, HU, JP, KG, KR, KZ, LK, LR, LT, LV, MD, MG, MN, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TO), ARIPO patent (KE, MW, SD, SZ). Published <i>With international search report.</i>
(54) Title: METHOD OF CONTROLLING PLANT PATHOGENS (57) Abstract <p>Plants can be transformed to express glucose oxidase from <i>Aspergillus</i> sp. and be made resistant to bacterial and fungal pathogens. Optionally, the plants may also express invertase to increase the available glucose as a substrate for enzymatic activity.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KB	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

METHOD OF CONTROLLING PLANT PATHOGENS

FIELD OF THE INVENTION

This invention relates to a method of controlling plant pathogens by
5 a protein which is provided by genetically modifying the plant to produce
the protein, and to genes and plants useful in that method.

BACKGROUND OF THE INVENTION

It is well known that the enzymatic action of glucose oxidase is anti-
10 bacterial. In the presence of oxygen, glucose oxidase catalyzes the oxidation of glucose to δ -gluconolactone and hydrogen peroxide. The antibacterial mode of action is due to both the oxidative potential of hydrogen peroxide as well as the presence of the δ -gluconolactone, which is a known glycosyltransferase inhibitor.

15 The antibacterial effect of the products of this enzyme has resulted in its widespread use in the food industry, where it is considered a GRAS [generally recognized as safe] compound. As such, glucose oxidase is used to prevent bacterial spoilage of prepared foods. In medicine, it is used as an enzymatic bactericide as part of a preparation for use in wound
20 dressings, toothpicks, dental floss and miniature toothbrushes. Glucose oxidase has also been mentioned as a method to control dental caries.

Recent reports have shown that a glucose oxidase is involved as part of the biocontrol mechanism used by *Penicillium dangearii* to control the plant pathogenic fungus *Verticillium dahliae*. [Kim et al., 1988, 1990].

25 However, the use of glucose oxidase as a means for plants to protect themselves from pathogenic organisms has been thought to have little potential due to the nature of the enzymatic action. First, there is little free glucose present in plants. The enzyme would seem to have insufficient substrate to produce enough hydrogen peroxide and/or δ -gluconolactone to
30 overcome a pathogenic attack. Second, the presence of such an enzyme in a plant cell, consuming glucose and producing even a small amount of hydrogen peroxide, would be expected to be detrimental to the vitality of the cell. Transgenic plants expressing glucose oxidase would not be expected to develop normally, either as regenerated plants or in subsequent generations.
35

It is an object of the present invention to provide a glucose oxidase than can be safely expressed in plant cells and provide disease resistance

-2-

to those cells. It is a further object of the present invention to provide a method of transforming plants to express a glucose oxidase which can be safely expressed in plants and provide disease resistance to those plants.

5

SUMMARY OF THE INVENTION

Surprisingly, it has been found that the gene for glucose oxidase from *Aspergillus niger* can be used to transform plants, which are developmentally normal and resist pathogenic attack. It is, therefore, an object of the present invention to provide genetic constructs comprising a gene for *Aspergillus* glucose oxidase (AGO) useful for insertion into plant cells. It is another object of the present invention to provide transformed, pathogen-resistant plants containing such genetic material.

Additionally, the plants may also be transformed to co-express other antifungal proteins or insecticidal proteins, for example, using *Bacillus thuringiensis* (*B.t.*) genes. Examples of plants transformed to express *B.t.* genes are disclosed in European Patent Publication No. 0 385 962, which corresponds to U.S. Serial Number 07/476,661, filed February 12, 1990 [Fischhoff et al.], which is incorporated herein by reference. A *B.t.* gene may be incorporated into a plant of the present invention by simultaneous transformation, sequential transformation, or by breeding.

In accordance with an aspect of the present invention, there is provided a recombinant, double-stranded DNA molecule comprising in operative sequence:

- a) a promoter which functions in plant cells to cause the production of an RNA sequence; and
- b) a structural coding sequence that codes for production of AGO;
- c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence.

In accordance with another aspect of the present invention, there is provided a method of producing genetically transformed plants which express an antipathogenic amount of AGO, comprising the steps of:

- a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising

-3-

- (i) a promoter which functions in plant cells to cause the production of an RNA sequence;
- (ii) a structural coding sequence that codes for production of AGO;
- 5 (iii) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;
- b) obtaining transformed plant cells; and
- c) regenerating from the transformed plant cells genetically transformed plants which express an inhibitory amount of AGO.
- 10

There is also provided, in accordance with another aspect of the present invention, transformed plants that contain DNA comprised of the above-mentioned elements (i), (ii), and (iii).

15 As used herein, the term "Aspergillus glucose oxidase" or "AGO" is used to indicate a glucose oxidase naturally produced by *Aspergillus* sp. or having $\geq 80\%$ homology, preferably $\geq 90\%$, to such an enzyme, for example, the enzyme encoded by SEQ ID NO: 1.

20 As used herein, the term "controlling microbial damage" or "pathogen-resistance" is used to indicate causing a reduction in damage to a crop due to infection by a bacterial or fungal pathogen.

As used herein, the term "structural coding sequence" means a DNA sequence which encodes for a polypeptide, which may be made by a cell following transcription of the DNA to mRNA, followed by translation to the desired polypeptide.

25

As used herein, the term "plant locus" means the area immediately surrounding a plant and including the plant and its root zone.

DETAILED DESCRIPTION OF THE INVENTION

30 One embodiment of the present invention comprises a protein isolated from *Aspergillus niger*. This protein, designated AGO, has been purified to homogeneity. It inhibits the growth of the agronomically important fungal pathogens, including, *Verticillium dahliae*, one of the most widespread and damaging plant pathogens, causing disease in many plants,

35 *Phytophthora infestans* (Pi), the causal pathogen of late blight disease in

-4-

potato and tomato, *Botrytis cinerea* (Bc), the source of gray mold on various fruits and vegetables, *Septoria nodorum* (Sn), the causal agent of wheat glume blotch, *Pseudocercospora herpotrichoides* (Ph), the causal agent of wheat eyespot, and *Gaeumannomyces graminis* var *tritici* (Ggt),
5 the causal agent of Take-all disease in cereals, with an amount as little as 50 ng under the assay conditions. It has also been found to inhibit *Erwinia carotovora*, the causal agent of potato soft rot, a post-harvest disease of potatoes. It is expected to be capable of controlling many other plant pathogenic organisms based on the products of its enzymatic activity, D-glucanolactone and hydrogen peroxide. Each of these byproducts is toxic to
10 such organisms.

Many species of plants may be protected by the methods of the present invention. For example, many fruits and vegetables such as strawberries, potatoes, and tomatoes may be protected from plant pathogens by
15 the present methods. Various *Phytophthora* species are pathogenic to many other plants, such as fruit trees or turf, and thus these plants may also be protected by the methods of the present invention. Furthermore, wheat and barley plants may be protected from Ggt, Sn, and Ph, by the present method.

20 As noted above, the antimicrobial proteins of the present invention may be used in combination with other antifungal proteins so as to provide a broad spectrum of activity, i.e., control additional pathogens, and/or provide multiple modes of action for the inhibition of the same fungal pathogen. Sources of such other antifungal proteins might be microbial, such as
25 the proteins of the present invention, or may be plants. Many such antifungal genes are reported in the literature.

Although AGO will function to protect plants from pathogenic attack in the presence of naturally occurring levels of glucose, it may be desirable to provide an invertase which will cause the hydrolysis of sucrose,
30 thus releasing additional glucose for the AGO to act on. The invertase will preferably be a cell wall invertase such as from yeast (EP 0 442 592, Willmitzer et al., 1991, also AU 70898/91) or a vacuolar enzyme such as from tomato or other plants. In these two cases the native signal sequences may be used; however, it may be preferable that the invertase
35 be sequestered until needed in the extracellular space, or not produced until

-5-

needed. The first alternative may be accomplished by the use of a signal sequence which will direct the enzyme to the extracellular space. One such signal is the potato protease inhibitor signal (Keil et al., 1986; Nelson et al., 1980). A promoter that is only active as a result of a pathogenic infection would be useful in limiting the expression of invertase to when it is actually needed to produce glucose as a substrate for AGO.

During storage, when *Erwinia* infection can cause the loss of a whole bin, potato tubers will naturally contain glucose from the breakdown of starch. Therefore, inclusion of a gene for invertase is not desired or necessary for protection from soft rot.

IN VITRO BIOEFFICACY ASSAYS

Antifungal assays

Glucose oxidase from *Aspergillus niger* can be obtained from Sigma Chemical Co. (St. Louis, Cat.# G-7141). It was used to test the *in vitro* activity against several organisms.

Tests against Pi and Bc were conducted in Medium #303, prepared as follows: One liter contains 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 2 g KH_2PO_4 ; 0.5 g NaCl; 1 g CaCO_3 ; 1 ml $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ stock - 1 mg/ml; 1 ml $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ stock - 1 mg/ml; 0.5 ml FeEDTA stock - 100 mM; 20 g Maltrin M-100; 20 g Casein; 5 g Yeast Extract; 5 g Glucose; 3.02 g Pipes 10 mM; pH adjusted to 6.5 and filter sterilized. Tests against Ggt were conducted in half-strength PDA (Difco). Tests against Ph were conducted in CDAA .1% media prepared as follows: 35 g/l Difco Czapek Dox Broth, 1 g/l Proline, 500 mg/l Asparagine, 500 mg/l Cysteine, and 1 g/l Agar are autoclaved for 23 minutes, and filter-sterilized vitamins (1 ppm Thiamine and 1 ppm Biotin) are added.

Bc and Pi were tested in a liquid assay in 96-well plates. Bc was used at 5×10^2 spores per well and allowed to incubate at 20 °C. for 24-48 hours. Pi is seeded at 5×10^3 sporangia per well and allowed to incubate at 18 °C for 24-48 hours. Assessment of growth is made by measuring the OD at 595 nm. The growth of Pi was 90% inhibited at concentrations as low as 3×10^{-5} IU/ μl . The growth of Bc was 95% inhibited at 0.001 IU/ μl .

Activity against *Gaeumannomyces* was evaluated on solid agar plates. An approximately 0.5 cm² piece of agar supporting heavy fungal growth was placed in the center of a half-strength PDA plate and allowed

-6-

to grow out for several days at 22 °C. At 1 cm beyond the leading edge of growth a 0.5 cm diameter plug of agar was removed aseptically with a sterile cork borer. Sterile AGO stock was added directly to these wells. A visible zone of inhibition was apparent with amounts as low as 0.02

5 IU/well.

Tests against Ph were conducted in 96-well plates. A 14 day old culture of *Pseudocercospora herpotrichoides* var. *tritici* on water agar is used to make the spore suspension. A plate is flooded with 5-10 ml of CDAA .1% media and spores are mixed into the liquid media by swirling gently.

10 The concentrated spore suspension is drawn off with pipet and added to the total volume of CDAA required for the test, adjusting spore concentration to 100,000 spores/mL. Assay incubation is at 24° C in darkness.

The spore suspension is dispensed at 50 µl/well in a 96 well micro-titer plate. These plates are then placed in an incubator (10 hr/day light at 12 °C) for 24 hours prior to sample application. 50 µl of sample is added to the 50 µl of inoculum (prepared 24 hours earlier) resulting in a total well volume of 100 µl/treated well/replicate treatment. Assay plates are incubated for 48 hours and the results are determined by reading optical density (OD) with a BioRad microtiter plate reader model 3550 at a single wavelength of 595 nm. An OD reading is made at time zero (t_0) which is made immediately after sample application, and an OD reading is made at 48 hours after sample application (t_{48}). Fungal growth estimate is determined by the difference in OD readings between t_0 and t_{48} multiplied by a calculation value for fungal biomass. (The calculation value for fungal biomass is the relationship between fungal growth and optical density and was determined in separate experiments. The relationship between fungal growth and optical density was determined by growing fungi in 96 well microtiter plates, and harvesting the mycelium over time, at absorbance intervals of approximately 0.1 OD. The calculation value comes from the linear relationship between fungal biomass and OD for the specific fungus. It is the slope value obtained from the linear relationship. The calculation value for Ph is 4.91) Then % inhibition is determined from the difference between the biomass of the treatments and the biomass of the controls. AGO exhibited 60% inhibition of Ph at 1.7×10^{-4} IU/µl.

35 Tests against Sn were conducted essentially like those for Ph

-7-

except for the spore suspension preparation. A seven day old sporulating culture of *Septoria nodorum* on YMA agar is used to make the spore suspension. A small amount (<1 ml) of CDAA media is dropped onto an area of the culture with pink spore masses oozing from the pycnidia. The spores are mixed with the CDAA media by repeatedly drawing up and expelling them from the pipetter. The concentrated suspension is added to the total volume of CDAA required for the test, adjusting spore concentration to 50,000 spores/ml. The calculation value for Sn is 0.508. AGO exhibited 60% inhibition of Sn at 1.7×10^{-4} IU/ μ l.

Bacterial Assay

Cultures of the bacteria *Erwinia carotovora* are maintained by streaking onto PDA plates and incubating at 24 °C in the dark. To prepare inoculum broth, a loop of actively growing bacteria (5-9 days old) is added to 50 ml of 1/4 strength PD broth in a 125 ml Erlenmeyer flask. The flask is placed on a shaker incubator (130 rpm) at 24 °C in the dark. After 24 hr, the bacteria are pelleted, resuspended in sterile deionized water, and three or four 100 μ l aliquots are each placed in wells of a 96-well microtiter plate for readings. The microplate reader is set at 595 nm, and the average optical density of the wells is determined. This absorbance (ABS = optical density) number is used in the following formula to calculate colony forming units (CFUs) contained in the broth.

$$\text{CFU} = (3 \times 10^6) + [(3 \times 10^8) \times \text{ABS}] + [(5 \times 10^8) \text{ABS}^2]$$

The broth is adjusted for use to 10^5 CFU.

AGO completely controlled growth of *E. carotovora* at concentrations as low as 3×10^{-5} IU/ μ l.

ENZYME IDENTIFICATION

A number of methods may be devised to detect the production of a protein in a heterologous system such as plant cells. Western blot techniques may be used to detect a protein immunologically, or enzymatic or biological assays may be used to detect activity of the protein.

Glucose Oxidase Enzymatic Assay

A modification of a continuous spectrophotometric assay

-8-

(Frederick *et al.*, 1990) was utilized to establish GO activity. *Aspergillus niger* GO (Sigma) was used as a positive control. The reaction mixture consisted of 20 µg/ml horseradish peroxidase (Sigma), 0.32 mM Triton X-100-stabilized *o*-dianisidine solution (Sigma), and 0.1 M glucose in 75 mM sodium phosphate buffer (pH 5.0). To this was added 100 µl of various concentrations of *A. niger* GO for the control reactions or 100 µl of sample derived from a heterologous source. The assay was performed at room temperature and the increase in absorbance was monitored at 460 nm.

An alternate assay for glucose oxidase utilizing the reagent 4-amino-antipyrine (4-AAP) was optimized for use based on Gallo, 1981. The 5X 4-AAP reagent is prepared at 10 ml total volume with 0.68 g KH_2PO_4 , 8.3 mg 4-AAP (0.82 mM), 25 µl Triton X-100, 0.0658 g crystalline phenol, and 1000 U Horseradish peroxidase, with the pH adjusted to 5.0 with KOH. The assay is conducted by mixing 200 µl of this 4-AAP reagent, 5 µl 1 mM FAD, 50 µl 1 M glucose; and the test sample (up to 750 µl). The result is read at OD508.

Glucose Oxidase Biological Activity Assay

A plug of 4-6 day old Ggt fungus was transferred onto fresh one-quarter-strength potato dextrose agar (Difco) plates. The fungus was grown at 22 °C for four days or until the circle of growth was about 2.5 cm. Using a sterile cork borer, wells were made in agar at 1 cm outside the circle of growth and 100 µl of buffer or sample derived from a heterologous source was placed in the wells. The fungus was grown for 24 hr at room temperature and examined for inhibition of growth.

Immunological Detection of Glucose Oxidase

Polyclonal antibodies to glucose oxidase produced by *Aspergillus niger* are available commercially from many sources, for example, Rockland, Inc., Gilbertsville, Pennsylvania.

GENETIC TRANSFORMATION

Cloning of the AGO Gene

Total DNA was isolated from *Aspergillus niger* (ATCC 9029) and used as a template for PCR isolation of a glucose oxidase gene. PCR

-9-

primers were based on the published sequence of the gene (Frederick et al; Kriechbaum et al.) and were designed to isolate the entire sequence of the gene including the signal sequence. In addition, in order to facilitate the incorporation of this gene into vectors appropriate for expression in heterologous bacterial, baculovirus or plant systems, the 5' PCR primer (SEQ ID NO:3) introduced XbaI and BglII restriction endonuclease sites upstream of the ATG start of translation of the gene. The 3' PCR primer (SEQ ID NO:4) introduced the BamHI and KpnI restriction endonuclease sites immediately after the stop codon. The PCR fragment produced was cloned into pUC118 as a XbaI/KpnI fragment to create pMON22514 and was completely sequenced. The sequence (SEQ ID NO:1) exactly matched that of the published sequence. SEQ ID NO:2 is the corresponding amino acid sequence.

15 Plant Gene Construction

The expression of a plant gene which exists in double-stranded DNA form involves transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which adds polyadenylate nucleotides to the 3' end of the RNA. Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter." The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding strand of RNA.

A number of promoters which are active in plant cells have been described in the literature. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the cauliflower mosaic virus (CaMV) 19S and 35S promoters, the Figwort Mosaic Virus (FMV) 35S promoter, and the light-inducible promoter from the small subunit of ribulose 1,5-bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide). All of these promoters have been used to create various types of DNA constructs which have been expressed in plants.

35 U.S. Patent Number 5,034,322 (Fraley et al., 1991), herein incorporated

-10-

by reference, discloses such uses.

There are promoters known which will limit expression to particular plant parts or in response to particular stimuli. For example, potato tuber specific promoters, such as the patatin promoters or the promoters for the large or small subunits of ADPglucose pyrophosphorylase, could be used to obtain expression primarily in the tuber and thus, in combination with AGO, provide resistance to attacks on the tuber, such as by *Erwinia*. A fruit specific promoter would be desirable to impart resistance to *Botrytis* in strawberries or grapes. A root specific promoter would be desirable to obtain expression of AGO in wheat or barley to provide resistance to Ggt. One skilled in the art will know of many such plant part-specific promoters which would be useful in the present invention.

Alternatively, the promoters utilized in the double-stranded DNA molecules may be selected to confer specific expression of AGO in response to fungal infection. The infection of plants by fungal pathogens triggers the induction of a wide array of proteins, termed defense-related or pathogenesis-related (PR) proteins [Bowles; Bol et al.; Linthorst]. Such defense-related or PR genes may encode phenylpropanoid metabolism enzymes (such as phenylalanine ammonia lyase, chalcone synthase, 4-coumarate coA ligase, coumaric acid 4-hydroxylase), proteins that modify plant cell walls (such as hydroxyproline-rich glycoproteins, glycine-rich proteins, peroxidases), enzymes (such as chitinases and glucanases) that degrade the fungal cell wall, thaumatin-like proteins, or proteins of as yet unknown function. The defense-related or PR genes have been isolated and characterized from a number of plant species. The promoters of these genes may be used to attain expression of AGO in transgenic plants when challenged with a pathogen, particularly a fungal pathogen such as Pi. Such promoters may derive from defense-related or PR genes isolated from potato itself [Fritzemeier et al.; Cuypers et al.; Logemann et al.; Matton and Brisson; Taylor et al.; Matton et al.; Schroder et al.]. In order to place the AGO gene under the control of a promoter induced by infection with *P. infestans* the promoter reported by Taylor et al. (1990) may be preferred.

The particular promoter selected should be capable of causing sufficient expression of the enzyme coding sequence to result in the production of an effective amount of AGO.

-11-

The promoters used in the DNA constructs (i.e. chimeric plant genes) of the present invention may be modified, if desired, to affect their control characteristics. For example, the CaMV 35S promoter may be ligated to the portion of the ssRUBISCO gene that represses the expression of ssRUBISCO in the absence of light, to create a promoter which is active in leaves but not in roots. The resulting chimeric promoter may be used as described herein. For purposes of this description, the phrase "CaMV 35S" promoter thus includes variations of CaMV 35S promoter, e.g., promoters derived by means of ligation with operator regions, random or controlled mutagenesis, etc. Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay et al.

An enhanced CaMV 35S promoter has been constructed as follows. A fragment of the CaMV 35S promoter extending between position -343 and +9 was previously constructed in pUC13. [Odell et al.] This segment contains a region identified as being necessary for maximal expression of the CaMV 35S promoter. It was excised as a ClaI-HindIII fragment, made blunt ended with DNA polymerase I (ClaI fragment) and inserted into the HincII site of pUC18. This upstream region of the 35S promoter was excised from this plasmid as a HindIII-EcoRV fragment (extending from -343 to -90) and inserted into the same plasmid between the HindIII and PstI sites. The enhanced CaMV 35S promoter (hereafter "CaMV E35S") thus contains a duplication of sequences between -343 and -90. [Kay et al.]

The RNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNA's, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. Rather, the non-translated leader sequence can be derived from an unrelated promoter or coding sequence. For example, the petunia heat shock protein 70 (Hsp70) contains such a leader. [Winter]

-12-

As noted above, the 3' non-translated region of the chimeric plant genes of the present invention contains a polyadenylation signal which functions in plants to cause the addition of adenylate nucleotides to the 3' end of the RNA. Examples of preferred 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene and (2) plant genes like the soybean 7s storage protein genes and the pea ssRUBISCO E9 gene. [Fischhoff, et al.]

10 Plant Transformation and Expression

A chimeric plant gene containing a structural coding sequence of the present invention can be inserted into the genome of a plant by any suitable method. Suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, e.g., by Herrera-Estrella (1983), Bevan (1983), Klee (1985) and EPO publication 0 120 516 (Schilperoort et al.). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA constructs of this invention into plant cells. Such methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, free DNA delivery via microprojectile bombardment, and transformation using viruses or pollen.

Plants which may be protected by the present invention include, but are not limited to, potatoes, tomatoes, wheat, corn, strawberries, grapes, as well as various ornamentals. Methods of tissue culture and plant regeneration are available for all of these types of plants.

Transient Expression of Glucose Oxidase in Tobacco Plants

A particularly useful plasmid cassette vector for transformation of dicotyledonous plants is pMON999. The expression cassette pMON999 consists of the CaMV E35S promoter, and the 3' end including polyadenylation signals from the NOS gene. pMON999 includes BglII, KpnI and EcoRI sites for insertion of coding sequences and NotI-NotI sites flanking the plant gene expression cassette.

35 The BglII/KpnI fragment of pMON22514 containing the AGO

-13-

coding region was inserted into pMON999 to create pMON22515. pMON22515 was electroporated into tobacco protoplasts. Expression of glucose oxidase by the transformed tobacco cells was confirmed by Western blot analysis as well as enzymatic assays.

5

Stable Transformation of Dicots

The shuttle vector for AGO was created by excising the AGO coding region as a BamHI/BglII fragment from pMON22515. It was then placed in a pUC-based vector, and the resulting plasmid, pMON22579, contains the FMV promoter, the AGO coding region, and the 3' end including polyadenylation signals from the NOS gene. The NotI/NotI fragment from this vector containing the FMV promoter, the AGO coding region, and the NOS gene 3' end including the polyadenylation signal was moved into the NotI restriction site of pMON17227 (WO 92/0449, Barry et al.) to generate the final Ti vector, pMON22587.

15

As discussed above, an AGO gene can also be expressed in specific parts of a plant by using tissue-specific promoters. The patatin promoter expresses primarily in the tuber of the potato. A KpnI/XbaI fragment containing the AGO coding region was excised from pMON22514 and inserted into a pUC-based vector containing the patatin 1.0 promoter (Bevan et al., 1986) and the 3' end including polyadenylation signals from the NOS gene to create pMON22516. The NotI fragment of pMON22516 containing the patatin promoter, the AGO coding region and the NOS gene 3' end including the polyadenylation signal was then moved into the NotI restriction site of pMON17227, which was described above, to create the Ti plasmid vector, pMON22517.

20

25

These vectors may be introduced into disarmed *Agrobacterium* ABI and used to transform potato, tomato, or other explants in tissue culture. After selection for kanamycin or glyphosate resistance and plant regeneration, whole plants containing an AGO gene may be recovered. Expression of the glucose oxidase gene may be confirmed by Western blot analysis, enzyme assay, or bioassay.

30

Expression of AGO by Transformed Potato Plants

35

Protein was extracted from the tubers of potato plants which had

-14-

been transformed with the Ti plasmid vector pMON22517 and were examined for the presence of glucose oxidase by Western blot analysis. High levels of glucose oxidase expression were detected in some of the plants. These levels of expression were confirmed by enzymatic assay
 5 using the 4-amino antipyrine system. Total protein was extracted from several of these tubers by grinding in 25mM Phosphate buffer pH7.0 + 5mM EDTA + 100mM KCl. Protein was concentrated and washed with 12.5 mM Phosphate Buffer pH7.0.

The protein extracted from tubers expressing AGO was tested
 10 against *P.infestans* in the 96-well plate assay in 12.5 mM Phosphate Buffer pH7.0. The results are shown in Table 1. At the highest level of protein tested, 21.2 µg/µl, Pi spores either remained ungerminated or growth from the spores was severely stunted when compared to the Hollow Vector (pMON17227) or Buffer control plant extracts.

15	<u>Treatment</u>	<u>Table 1</u>
	<u>Fungal Inhibition*</u>	
	Buffer control	0
	Hollow vector control	2
	pMON22517, line 9	3
20	pMON22517, line 30	3

*Scale: 0=no inhibition, 1=slight inhibition, 2=moderate inhibition, 3=severe inhibition.

Leaves of potato plants transformed with the Ti plasmid vector pMON22587 were tested for the presence of AGO by means of an
 25 enzymatic assay. The results are given in Table 2. As above, pMON17227-transformed potato plants were used as hollow vector control plants.

30	<u>Line #</u>	<u>Expression</u>	<u>Glucose oxidase</u>	<u>Equivalent</u>
		level ^a	activity ^b	to ^c
		<u>(%)</u>	<u>(U/10 ng X 10⁻⁴)</u>	<u>(U/gFW)</u>
	Rus. Bur.	0	0	0
	22587-2	0.075	72.2	7.22
	22587-3	0.150	126.4	12.64
	22587-4	0.007	6.0	0.6
35	22587-12	0.125	88.2	8.82

-15-

22587-31	0.350	140.4	14.04
22587-32	0.350	121.0	12.10
22587-33	0.450	181.8	18.18
22587-34	0.003	1.2	0.12

- 5 a Expression level is indicated as the percentage of glucose oxidase in total extractable leaf protein.
- b Glucose oxidase activity in leaf extracts were determined by enzyme activity assay and calculated as units of the enzyme activity in total protein.
- 10 c Units of glucose oxidase activity per gram fresh weight of leaf tissue (U/gFW) were calculated from the activity in total protein by the conversion factor of 10 µg total protein per gram fresh weight of leaf tissue.

- 15 The leaves of potato plants transformed with the Ti plasmid vector pMON22587 were also tested for levels of H₂O₂ by titanium chloride precipitation. In two lines the leaves were found to have levels of H₂O₂ two- to four-fold higher than the leaves of nontransformed control plant.

20 Disease-Resistant Potatoes

- Tubers expressing AGO were tested against *Erwinia carotovora* in a tuber disc assay. Tubers from 34 lines transformed with pMON22517 were tested for resistance to soft rot lesions. The lines were also tested for expression levels by Western blot analysis. The tubers were surface steri-
- 25 lized according to the method of Yang et al. (1989). Two or three tubers per line were aseptically cut into discs 7-10mm thick, resulting in six to twelve discs per line. The discs were placed on moistened sterile Whatman #1 filter paper in petri dishes. The center of each disc was inoculated with 50,000 CFU of bacterial in 10 µl. The dishes were maintained in a high
- 30 humidity environment for 3 days at 23 °C. Disease evaluations included measuring the length, radius, and depth of the resulting lesions. Dilutions of macerated tissue from within the lesion were used to determine the final number of bacteria per lesion. The results of this determination are presented in Table 3. For the lines that appeared to be the most protected, all of
- 35 the measured parameters were better than those of the Hollow Vector and

-16-

Buffer control tubers. Tubers found to be expressing AGO at the highest level generally had the lowest level of bacteria in the lesions.

Table 3

		Expression ^b	
5	<u>LINE #</u>	<u>Levels</u>	<u>CFU X 10²</u>
	Buffer Control	null	16.76
	Hollow vector	null	14.82
	22517-10	null	20.7
	22517-24	null	20.09
10	22517-13	null	16.3
	22517-12	null	19.26
	22517-9	LOW	9.34
	22517-28	LOW	13.56
	22517-33	LOW	9.57
15	22517-14	LOW	8.22
	22517-17	LOW	8.36
	22517-19	LOW	9.37
	22517-32	LOW	7.17
	22517-20	LOW	7.4
20	22517-21	LOW	6.25
	22517-34	LOW	4.44
	22517-11	LOW	8.06
	22517-8	LOW	1.5
	22517-7	MEDIUM	12.88
25	22517-31	MEDIUM	12.06
	22517-1	MEDIUM	7.54
	22517-15	MEDIUM	6.7
	22517-25	MEDIUM	12.25
	22517-35	MEDIUM	6.81
30	22517-2	MEDIUM	3.52
	22517-3	MEDIUM	1.26
	22517-5	MEDIUM	1.4
	22517-6	HIGH	15.74
	22517-18	HIGH	2.675
35	22517-26	HIGH	4.55

-17-

	22517-29	HIGH	4.35
	22517-4	HIGH	4.2
	22517-30	HIGH	3.92
	22517-16	HIGH	1.12
5	22517-23	HIGH	2.67
	22517- 36	HIGH	0.855

^a CFU = colony forming units

^b Expression levels determined by Western Blot Analysis

- Leaflets from plants transformed with pMON22587 and expressing
 10 AGO were tested for resistance to Pi. Fully expanded leaflets (~20 cm²) were inoculated by adding droplets of 100 µl sporangium suspension of *Phytophthora infestans* to the center of the abaxial leaf surface. The inocula had a density of 10⁵-10⁸ sporangia per ml collected from 2-3 week old plates containing LB-V8 medium.. The inoculated leaflets were main-
 15 tained in Nunc Bio-Assay dishes (243 x 243 x 18 cm) with moisture provided by wet filter paper at the bottom, and incubated in growth chambers at ~19°C with 16 h photoperiod. The development of symptoms was observed and infected areas on the leaflets were measured by overlaying each leaflet with a 5 mm x 5 mm transparent grid. For each line of leaflets,
 20 the mean of infected areas and the standard deviation were calculated.

The transgenic lines expressing AGO showed significant control of the symptoms caused by *Phytophthora infestans* infection on the leaflets. The results of two lines are shown in Table 4. The symptom reduction was 47 and 57% as compared to controls (both nontransformed and hollow
 25 vector transformants).

Table 4

Area of Infection^a (cm²)

	<u>Line No.</u>	<u>3 dpi^b</u>	<u>4 dpi</u>	<u>5 dpi</u>	<u>6 dpi</u>
	Russet Burbank	1.2	2.1	4.2	7.1
30	17227-1 ^c	1.6	2.7	4.7	8.7
	22587-3 ^d	0.4	0.8	1.1	3.0
	22587-12 ^d	0.5	1.3	2.3	3.7

^a Area of infection is indicated as the mean of five leaflets.

^b Days post inoculation.

35 ^c A control line transformed with vector only.

-18-

d Transgenic lines expressing glucose oxidase.

Stable Transformation of Monocots

For transformation of monocots a vector was constructed with
5 AGO and an intron particularly useful in increasing frequency of obtaining
transformed plants which express a desired protein at high levels. The
hsp70 intron disclosed in EP 602 193 (equivalent to U.S. Serial Number
08/181,364, Brown et al., incorporated herein by reference) was used.
pMON19477, disclosed therein, was cut, and the 800 bp BglII-BamHI
10 fragment containing the hsp70 intron was then cloned into the unique BglII
site in pMON22515, resulting in pMON22623.

pMON22623 has been introduced into wheat cells by microprojectile bombardment using known methods (Vasil et al.)

When transformed plants are recovered, their fungal resistance
15 capacity, particularly to Ggt, will be assessed by known methods.

All publications and patents mentioned in this specification are
herein incorporated by reference as if each individual publication or patent
was specifically and individually stated to be incorporated by reference.

From the foregoing, it will be seen that this invention is one well
20 adapted to attain all the ends and objects hereinabove set forth together
with advantages which are obvious and which are inherent to the
invention.

It will be understood that certain features and subcombinations are
of utility and may be employed without reference to other features and
25 subcombinations. This is contemplated by and is within the scope of the
claims.

Since many possible embodiments may be made of the invention
without departing from the scope thereof, it is to be understood that all
matter herein set forth or shown in the accompanying drawings is to be
30 interpreted as illustrative and not in a limiting sense.

REFERENCES

- Barry, G.F., G. Kishore, and S.R. Padgett. "Glyphosate Tolerant 5-Enol-pyruvylshikimate-3-phosphate Synthases." PCT Appl. WO 92/04449, 1991.
- 5 Bevan, M. et al. Nature, 304:184, 1983.
Bevan, M. et al. Nucleic Acid Research, 14:4625, 1986.
Bol, J. et al. Ann Rev of Phytopathol., 28:113-138, 1990.
Bowles, D. Ann Rev of Biochem., 59:873-907, 1990.
Cuyppers, B. et al. Mol Plant-Microbe Interactions, 1:157-160, 1988.
- 10 Fischhoff, D.A. and Perlak, F.J. "Synthetic plant genes and method for preparation." European Patent Application, Publication Number 0 385 962, 1990.
- Frederick, K. et al. J. Biol. Chem., 265:3793-3802.
Fritzscheier, K. et al. Plant Physiol, 85:34-41, 1987.
- 15 Gallo. Methods in Enzymology, 71:665-668, 1981.
Herrera-Estrella, L. et al. Nature, 303:20-9, 1983.
Kay, R. et al., Science, 236:1299-1302, 1987.
Keil, M. et al. Nucleic Acids Res., 14:5641 - 5650, 1986.
Klee, H. J. et al. Bio/Technology, 3:637-642, 1985.
- 20 Kim, K. et al. Phytopathology, 78:488-492, 1988.
Kim, K. et al. Can. J. Microbiol., 36:199-205, 1990.
Kriechbaum M. et al. FEBS Lett., 255:63-66, 1989.
Linthorst, H. J. M. Crit Rev Plant Sci, 10:123-150.
Logemann, J. et al. Plant Cell, 1:151-158, 1989.
- 25 Matton, D. and Brisson, N. Mol Plant-Microbe Interactions, 2:325-331, 1989.
- Matton, D. et al. Plant Mol Biol, 14:863-865, 1990.
Nelson, C. et al. Proc. Natl. Acad. Sci. USA, 77:1975 - 1979, 1980.
Odell, J. et al., Nature, 313:810, 1985.
- 30 Schroder, M. et al. Plant J, 2:161-172, 1992.
Taylor, J., et al. Molecular Plant-Microbe Interactions, 3:72-77, 1990.
Vasil et al. Bio/Technology 11: 1153-1158, 1993.
Winter et al. Mol. Gen. Genet., 221(2):315-19, 1988.

-20-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Monsanto Company
(B) STREET: 800 North Lindbergh Boulevard
(C) CITY: St. Louis
(D) STATE: Missouri
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 63198
(G) TELEPHONE: (314)537-7286
(H) TELEFAX: (314)537-6047

(ii) TITLE OF INVENTION: Method of Controlling Plant Pathogens

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/161041
(B) FILING DATE: 24-NOV-1993

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1848 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 16..1833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTAGAAGAT CTATC ATG CAG ACT CTC CTT GTG AGC TCG CTT GTG GTC TCC	51
Met Gln Thr Leu Leu Val Ser Ser Leu Val Val Ser	
1 5 10	
CTC GCT GCG GCC CTG CCA CAC TAC ATC AGG AGC AAT GGC ATT GAA GCC	99
Leu Ala Ala Ala Leu Pro His Tyr Ile Arg Ser Asn Gly Ile Glu Ala	
15 20 25	

-21-

AGC CTC CTG ACT GAT CCC AAG GAT GTC TCC GGC CGC ACG GTC GAC TAC	147
Ser Leu Leu Thr Asp Pro Lys Asp Val Ser Gly Arg Thr Val Asp Tyr	
30 35 40	
ATC ATC GCT GGT GGA GGT CTG ACT GGA CTC ACC ACC GCT GCT CGT CTG	195
Ile Ile Ala Gly Gly Gly Leu Thr Gly Leu Thr Thr Ala Ala Arg Leu	
45 50 55 60	
ACG GAG AAC CCC AAC ATC AGT GTG CTC GTC ATC GAA AGT GGC TCC TAC	243
Thr Glu Asn Pro Asn Ile Ser Val Leu Val Ile Glu Ser Gly Ser Tyr	
65 70 75	
GAG TCG GAC AGA GGT CCT ATC ATT GAG GAC CTG AAC GCC TAC GGC GAC	291
Glu Ser Asp Arg Gly Pro Ile Ile Glu Asp Leu Asn Ala Tyr Gly Asp	
80 85 90	
ATC TTT GGC AGC AGT GTA GAC CAC GCC TAC GAG ACC GTG GAG CTC GCT	339
Ile Phe Gly Ser Ser Val Asp His Ala Tyr Glu Thr Val Glu Leu Ala	
95 100 105	
ACC AAC AAT CAA ACC GCG CTG ATC CGC TCC GGA AAT GGT CTC GGT GGC	387
Thr Asn Asn Gln Thr Ala Leu Ile Arg Ser Gly Asn Gly Leu Gly Gly	
110 115 120	
TCT ACT CTA GTG AAT GGT GGC ACC TGG ACT CGC CCC CAC AAG GCA CAG	435
Ser Thr Leu Val Asn Gly Gly Thr Trp Thr Arg Pro His Lys Ala Gln	
125 130 135 140	
GTT GAC TCT TGG GAG ACT GTC TTT GGA AAT GAG GGC TGG AAC TGG GAC	483
Val Asp Ser Trp Glu Thr Val Phe Gly Asn Glu Gly Trp Asn Trp Asp	
145 150 155	
AAT GTG GCC GCC TAC TCC CTC CAG GCT GAG CGT GCT CGC GCA CCA AAT	531
Asn Val Ala Ala Tyr Ser Leu Gln Ala Glu Arg Ala Arg Ala Pro Asn	
160 165 170	
GCC AAA CAG ATC GCT GCT GGC CAC TAC TTC AAC GCA TCC TGC CAT GGT	579
Ala Lys Gln Ile Ala Ala Gly His Tyr Phe Asn Ala Ser Cys His Gly	
175 180 185	
GTT AAT GGT ACT GTC CAT GCC GGA CCC CGC GAC ACC GGC GAT GAC TAT	627
Val Asn Gly Thr Val His Ala Gly Pro Arg Asp Thr Gly Asp Asp Tyr	
190 195 200	
TCT CCC ATC GTC AAG GCT CTC ATG AGC GCT GTC GAA GAC CGG GGC GTT	675
Ser Pro Ile Val Lys Ala Leu Met Ser Ala Val Glu Asp Arg Gly Val	
205 210 215 220	
CCC ACC AAG AAA GAC TTC GGA TGC GGT GAC CCC CAT GGT GTG TCC ATG	723
Pro Thr Lys Lys Asp Phe Gly Cys Gly Asp Pro His Gly Val Ser Met	
225 230 235	
TTC CCC AAC ACC TTG CAC GAA GAC CAA GTG CGC TCC GAT GCC GCT CGC	771
Phe Pro Asn Thr Leu His Glu Asp Gln Val Arg Ser Asp Ala Ala Arg	
240 245 250	

-22-

GAA TGG CTA CTT CCC AAC TAC CAA CGT CCC AAC CTG CAA GTC CTG ACC Glu Trp Leu Leu Pro Asn Tyr Gln Arg Pro Asn Leu Gln Val Leu Thr 255 260 265	819
GGA CAG TAT GTT GGT AAG GTG CTC CTT AGC CAG AAC GGC ACC ACC CCT Gly Gln Tyr Val Gly Lys Val Leu Leu Ser Gln Asn Gly Thr Thr Pro 270 275 280	867
CGT GCC GTT GGC GTG GAA TTC GGC ACC CAC AAG GGC AAC ACC CAC AAC Arg Ala Val Gly Val Glu Phe Gly Thr His Lys Gly Asn Thr His Asn 285 290 295 300	915
GTT TAC GCT AAG CAC GAG GTC CTC CTG GCC GCG GGC TCC GCT GTC TCT Val Tyr Ala Lys His Glu Val Leu Leu Ala Ala Gly Ser Ala Val Ser 305 310 315	963
CCC ACA ATC CTC GAA TAT TCC GGT ATC GGA ATG AAG TCC ATC CTG GAG Pro Thr Ile Leu Glu Tyr Ser Gly Ile Gly Met Lys Ser Ile Leu Glu 320 325 330	1011
CCC CTT GGT ATC GAC ACC GTC GTT GAC CTG CCC GTC GGC TTG AAC CTG Pro Leu Gly Ile Asp Thr Val Val Asp Leu Pro Val Gly Leu Asn Leu 335 340 345	1059
CAG GAC CAG ACC ACC GCT ACC GTC CGC TCC CGC ATC ACC TCT GCT GGT Gln Asp Gln Thr Thr Ala Thr Val Arg Ser Arg Ile Thr Ser Ala Gly 350 355 360	1107
GCA GGA CAG GGA CAG GCC GCT TGG TTC GCC ACC TTC AAC GAG ACC TTT Ala Gly Gln Gly Gln Ala Ala Trp Phe Ala Thr Phe Asn Glu Thr Phe 365 370 375 380	1155
GGT GAC TAT TCC GAA AAG GCA CAC GAG CTG CTC AAC ACC AAG CTG GAG Gly Asp Tyr Ser Glu Lys Ala His Glu Leu Leu Asn Thr Lys Leu Glu 385 390 395	1203
CAG TGG GCC GAA GAG GCC GTC GCC CGT GGC GGA TTC CAC AAC ACC ACC Gln Trp Ala Glu Glu Ala Val Ala Arg Gly Gly Phe His Asn Thr Thr 400 405 410	1251
GCC TTG CTC ATC CAG TAC GAG AAC TAC CGC GAC TGG ATT GTC AAC CAC Ala Leu Leu Ile Gln Tyr Glu Asn Tyr Arg Asp Trp Ile Val Asn His 415 420 425	1299
AAC GTC GCG TAC TCG GAA CTC TTC CTC GAC ACT GCC GGA GTA GCC AGC Asn Val Ala Tyr Ser Glu Leu Phe Leu Asp Thr Ala Gly Val Ala Ser 430 435 440	1347
TTC GAT GTG TGG GAC CTT CTG CCC TTC ACC CGA GGA TAC GTT CAC ATC Phe Asp Val Trp Asp Leu Leu Pro Phe Thr Arg Gly Tyr Val His Ile 445 450 455 460	1395
CTC GAC AAG GAC CCC TAC CTT CAC CAC TTC GCC TAC GAC CCT CAG TAC Leu Asp Lys Asp Pro Tyr Leu His His Phe Ala Tyr Asp Pro Gln Tyr 465 470 475	1443

-23-

TTC CTC AAC GAG CTG GAC CTG CTC GGT CAG GCT GCC GCT ACT CAA CTG Phe Leu Asn Glu Leu Asp Leu Leu Gly Gln Ala Ala Ala Thr Gln Leu 480 485 490	1491
CCC CGC AAC ATC TCC AAC TCC GGT GCC ATG CAG ACC TAC TTC GCT GGG Ala Arg Asn Ile Ser Asn Ser Gly Ala Met Gln Thr Tyr Phe Ala Gly 495 500 505	1539
GAG ACT ATC CCC GGT GAT AAC CTC GCG TAT GAT GCC GAT TTG AGC GCC Glu Thr Ile Pro Gly Asp Asn Leu Ala Tyr Asp Ala Asp Leu Ser Ala 510 515 520	1587
TGG ACT GAG TAC ATC CCG TAC CAC TTC CGT CCT AAC TAC CAT GGC GTG Trp Thr Glu Tyr Ile Pro Tyr His Phe Arg Pro Asn Tyr His Gly Val 525 530 535 540	1635
GGT ACT TGC TCC ATG ATG CCG AAG GAG ATG GGC GGT GTT GTT GAT AAT Gly Thr Cys Ser Met Met Pro Lys Glu Met Gly Gly Val Val Asp Asn 545 550 555	1683
GCT GCC CGT GTG TAT GGT GTG CAG GGA CTG CGT GTC ATT GAT GGT TCT Ala Ala Arg Val Tyr Gly Val Gln Gly Leu Arg Val Ile Asp Gly Ser 560 565 570	1731
ATT CCT CCT ACG CAA ATG TCG TCC CAT GTC ATG ACG GTG TTC TAT GCC Ile Pro Pro Thr Gln Met Ser Ser His Val Met Thr Val Phe Tyr Ala 575 580 585	1779
ATG GCG CTA AAA ATT TCG GAT GCT ATC TTG GAA GAT TAT GCT TCC ATG Met Ala Leu Lys Ile Ser Asp Ala Ile Leu Glu Asp Tyr Ala Ser Met 590 595 600	1827
CAG TGATAAGGAT CCGGTACC Gln 605	1848

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 605 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gln Thr Leu Leu Val Ser Ser Leu Val Val Ser Leu Ala Ala Ala 1 5 10 15
Leu Pro His Tyr Ile Arg Ser Asn Gly Ile Glu Ala Ser Leu Leu Thr 20 25 30
Asp Pro Lys Asp Val Ser Gly Arg Thr Val Asp Tyr Ile Ile Ala Gly 35 40 45

-24-

Gly Gly Leu Thr Gly Leu Thr Thr Ala Ala Arg Leu Thr Glu Asn Pro
 50 55 60
 Asn Ile Ser Val Leu Val Ile Glu Ser Gly Ser Tyr Glu Ser Asp Arg
 65 70 75 80
 Gly Pro Ile Ile Glu Asp Leu Asn Ala Tyr Gly Asp Ile Phe Gly Ser
 85 90 95
 Ser Val Asp His Ala Tyr Glu Thr Val Glu Leu Ala Thr Asn Asn Gln
 100 105 110
 Thr Ala Leu Ile Arg Ser Gly Asn Gly Leu Gly Gly Ser Thr Leu Val
 115 120 125
 Asn Gly Gly Thr Trp Thr Arg Pro His Lys Ala Gln Val Asp Ser Trp
 130 135 140
 Glu Thr Val Phe Gly Asn Glu Gly Trp Asn Trp Asp Asn Val Ala Ala
 145 150 155 160
 Tyr Ser Leu Gln Ala Glu Arg Ala Arg Ala Pro Asn Ala Lys Gln Ile
 165 170 175
 Ala Ala Gly His Tyr Phe Asn Ala Ser Cys His Gly Val Asn Gly Thr
 180 185 190
 Val His Ala Gly Pro Arg Asp Thr Gly Asp Asp Tyr Ser Pro Ile Val
 195 200 205
 Lys Ala Leu Met Ser Ala Val Glu Asp Arg Gly Val Pro Thr Lys Lys
 210 215 220
 Asp Phe Gly Cys Gly Asp Pro His Gly Val Ser Met Phe Pro Asn Thr
 225 230 235 240
 Leu His Glu Asp Gln Val Arg Ser Asp Ala Ala Arg Glu Trp Leu Leu
 245 250 255
 Pro Asn Tyr Gln Arg Pro Asn Leu Gln Val Leu Thr Gly Gln Tyr Val
 260 265 270
 Gly Lys Val Leu Leu Ser Gln Asn Gly Thr Thr Pro Arg Ala Val Gly
 275 280 285
 Val Glu Phe Gly Thr His Lys Gly Asn Thr His Asn Val Tyr Ala Lys
 290 295 300
 His Glu Val Leu Leu Ala Ala Gly Ser Ala Val Ser Pro Thr Ile Leu
 305 310 315 320
 Glu Tyr Ser Gly Ile Gly Met Lys Ser Ile Leu Glu Pro Leu Gly Ile
 325 330 335
 Asp Thr Val Val Asp Leu Pro Val Gly Leu Asn Leu Gln Asp Gln Thr
 340 345 350

-25-

Thr Ala Thr Val Arg Ser Arg Ile Thr Ser Ala Gly Ala Gly Gln Gly
 355 360 365
 Gln Ala Ala Trp Phe Ala Thr Phe Asn Glu Thr Phe Gly Asp Tyr Ser
 370 375 380
 Glu Lys Ala His Glu Leu Leu Asn Thr Lys Leu Glu Gln Trp Ala Glu
 385 390 395 400
 Glu Ala Val Ala Arg Gly Gly Phe His Asn Thr Thr Ala Leu Leu Ile
 405 410 415
 Gln Tyr Glu Asn Tyr Arg Asp Trp Ile Val Asn His Asn Val Ala Tyr
 420 425 430
 Ser Glu Leu Phe Leu Asp Thr Ala Gly Val Ala Ser Phe Asp Val Trp
 435 440 445
 Asp Leu Leu Pro Phe Thr Arg Gly Tyr Val His Ile Leu Asp Lys Asp
 450 455 460
 Pro Tyr Leu His His Phe Ala Tyr Asp Pro Gln Tyr Phe Leu Asn Glu
 465 470 475 480
 Leu Asp Leu Leu Gly Gln Ala Ala Ala Thr Gln Leu Ala Arg Asn Ile
 485 490 495
 Ser Asn Ser Gly Ala Met Gln Thr Tyr Phe Ala Gly Glu Thr Ile Pro
 500 505 510
 Gly Asp Asn Leu Ala Tyr Asp Ala Asp Leu Ser Ala Trp Thr Glu Tyr
 515 520 525
 Ile Pro Tyr His Phe Arg Pro Asn Tyr His Gly Val Gly Thr Cys Ser
 530 535 540
 Met Met Pro Lys Glu Met Gly Gly Val Val Asp Asn Ala Ala Arg Val
 545 550 555 560
 Tyr Gly Val Gln Gly Leu Arg Val Ile Asp Gly Ser Ile Pro Pro Thr
 565 570 575
 Gln Met Ser Ser His Val Met Thr Val Phe Tyr Ala Met Ala Leu Lys
 580 585 590
 Ile Ser Asp Ala Ile Leu Glu Asp Tyr Ala Ser Met Gln
 595 600 605

(2) INFORMATION FOR SEQ ID NO:3:

- (1) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

-26-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCATCTAGAA GATCTATCAT GCAGACTCTC CTT

33

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGGGGTACCG GATCCTTATC ACTCCATGGA AGCATA

36

WHAT IS CLAIMED IS:

1. A recombinant, double-stranded DNA molecule comprising in operative sequence:
 - 5 a) a promoter which functions in plant cells to cause the production of an RNA sequence;
 - b) a structural coding sequence that encodes for production of AGO; and
 - 10 c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence.
2. The DNA molecule of Claim 1 wherein said structural DNA sequence is SEQ ID NO:1.
3. The DNA molecule of Claim 1 wherein said promoter is selected from FMV35S and CaMV35S promoters.
- 15 4. The DNA molecule of Claim 1 wherein said promoter is induced by a pathogenic infection.
5. A method of producing genetically transformed, disease resistant plants, comprising the steps of:
 - 20 a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising
 - (i) a promoter which functions in plant cells to cause the production of an RNA sequence;
 - (ii) a structural coding sequence that causes the production of AGO;
 - 25 (iii) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;
 - 30 b) obtaining transformed plant cells; and
 - c) regenerating from the transformed plant cells genetically transformed plants which express AGO in an amount effective to reduce damage due to infection by a bacterial or fungal pathogen..
6. The method of Claim 5 wherein said structural coding sequence is SEQ ID NO:1.
- 35

-28-

7. The method of Claim 5 wherein said promoter is selected from FMV35S and CaMV35S promoters.
8. The method of Claim 5 wherein said promoter is induced by pathogen infection.
- 5 9. The method of Claim 5 wherein said plants are potato or wheat plants.
10. A genetically transformed, disease resistant plant comprising a recombinant, double-stranded DNA molecule comprising in operative sequence:
 - 10 a) a promoter which functions in plant cells to cause the production of an RNA sequence;
 - b) a structural coding sequence that encodes for production of AGO; and
 - 15 c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence.
11. The plant of Claim 10 wherein said promoter is selected from FMV35S and CaMV35S promoters.
12. The plant of Claim 10 wherein said promoter is induced by pathogen infection.
- 20 13. The plant of Claim 10 wherein said structural coding sequence is SEQ ID NO:1.
14. The plant of Claim 10 which is a potato or wheat plant.

INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/US 94/11837

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/82 C12N15/53 A01N63/00 A01H5/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A01N A01H		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CANADIAN JOURNAL OF MICROBIOLOGY, vol.36, no.11, November 1990 pages 760 - 764 KIM, K., ET AL. 'Glucose oxidase as the antifungal principle of talaron from Talaromyces flavus' see the whole document ---	1-14
A	PHYTOPATHOLOGY, vol.78, no.4, 1988 pages 488 - 492 KIM, K.K., ET AL. 'Identification of a metabolite produced by Talaromyces-flavus as glucose oxidase and its role in the biocontrol of Verticillium-dahliae' see the whole document --- -/-	1-14
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 9 February 1995		Date of mailing of the international search report 17.02.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016		Authorized officer Maddox, A

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/11837

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,88 02600 (POULSEN) 21 April 1988 see the whole document	1-14
A	WO,A,86 04213 (PHARMACIA) 31 July 1986 see page 9, paragraph 2	1-14
A	WO,A,91 06217 (CULTOR) 16 May 1991 see the whole document	1-14
A	PHYTOPATHOLOGY, vol.82, no.6, 1992 pages 696 - 699 PENG, M., ET AL. 'Peroxidase-generated hydrogen peroxide as a source of antifungal activity in-vitro and on tobacco leaf disks' see the whole document	1-14
A	WO,A,93 11671 (GENENCOR) 24 June 1993 see the whole document	1-14
A	WO,A,89 12675 (CHIRON) 28 December 1989 see the whole document	1-14
A	NATURE, vol.361, 14 January 1993 pages 153 - 156 HAIN, R., ET AL. 'Disease resistance results from foreign phytoalexin expresion in a novel plant' see the whole document	1-14

INTERNATIONAL SEARCH REPORT
information on patent family members

International Application No
PCT/US 94/11837

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8802600	21-04-88	AU-A- 8175087	06-05-88
		EP-A- 0293407	07-12-88
		JP-T- 1501000	06-04-89
WO-A-8604213	31-07-86	EP-A- 0210250	04-02-87
		SE-A- 8500341	25-07-86
WO-A-9106217	16-05-91	AT-T- 109623	15-08-94
		CA-A- 2071544	28-04-91
		DE-D- 69011525	15-09-94
		DE-T- 69011525	01-12-94
		EP-A- 0497812	12-08-92
WO-A-9311671	24-06-93	AU-B- 3244993	19-07-93
		EP-A- 0618769	12-10-94
WO-A-8912675	28-12-89	US-A- 5094951	10-03-92
		EP-A- 0379556	01-08-90
		JP-T- 3500008	10-01-91
		US-A- 5266688	30-11-93